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(54) Title: MODIFIED NUCLEOTIDES

(57) Abstract

The present invention provides novel modified nucleotide compounds having the general formula: X-n(J)-(d or r)NTP where N is adenosine, guanosine or cytidine; X is H, a fluorophore, a chromophore, a luminescent compound, a ligand or a hapt- en; n is an integer of 7 or more; and J is a Jeffamine (TM Texaco, Inc.) substituent. These compounds are substantially superior substrates for incorporating label into nucleic acids during enzyme-catalyzed synthesis. Use of a Jeffamine-linked labelled nucle- otide derivative as a partial substitute for unlabelled substrate therefore leads to significantly greater incorporation, hence in- creased label density per unit length of nucleic acid, than the same label joined by a prior art linker. Jeffamine-modified ribo- or deoxyribo-nucleotides are also provided for synthesis of Jeffamine-modified nucleic acids. The invention further provides an im- proved method of synthesizing labelled nucleic acids. This method provides greater frequency and higher efficiency of label in- corporation, thus requiring lower amounts of nucleotide derivative in the reaction mixture.

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MODIFIED NUCLEOTIDES

BACKGROUND AND PRIOR ART

5 The invention concerns novel compounds for nucleic acid labelling and methods for making nucleic acids incorporating the novel compounds.

10 Techniques for labelling nucleic acids with a reporter molecule generally fall into one of two categories: incorporating the label into the nucleic acid during synthesis, or post-synthetically modifying the nucleic acid. The former is commonly accomplished by providing a labelled derivative of one of the precursor nucleotide triphosphates as a partial or complete substitute for the normal precursor during the enzyme-catalyzed synthesis of the nucleic acid. The labelled nucleotide derivative must meet certain criteria. The derivative must continue to be recognized by the enzyme as a substrate, it must not interfere with or inhibit the enzyme and it must participate in the normal hydrogen-bonding interactions of base pairing, adenine with thymine (or uracil), guanine with cytosine.

20 Post-synthetic modification labelling is commonly accomplished by modifying the end group of a nucleic acid by means of a chemical reaction or by an enzyme such as terminal transferase.

25 A wide variety of reporter molecules have been incorporated into nucleic acids. Besides radioactive labels, which are usually incorporated during synthesis, fluorescent labels, chromatic labels, luminescent labels, ligands and haptens have

been employed. Rhodamine and fluorescein have been used for fluorescent labelling. Nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate) (Gibco BRL) have been used as chromophores. Firefly luciferin and PPD (4-methoxy-4-(3-phosphatenehenyl)spiro[1,2 dioxetane-3,2'-adamantane] (Gibco BRL) have been used as luminescent labels. Biotin has been used as a ligand to bind labelled streptavidin. Dinitrophenol and digoxigenin have been used as hapten labels to bind antibody and take advantage of immunoassay methods. The foregoing examples are illustrative only and not limiting.

Radioactive labels have the advantage of providing high sensitivity, however they have the disadvantages of being expensive, of having short shelf life in some cases, and of presenting safety and disposal problems. While non-radioactive labels lack the disadvantages of radioactivity, they pose other difficulties for the potential user. Many of the reporter compounds are large, bulky molecules relative to the nucleotides themselves, and their size can sterically interfere with incorporation. Low levels of incorporation cause loss of detection sensitivity by limiting the level of detectable signal per nucleic acid molecule. The reaction conditions used for post-synthetic nucleic acid labelling can be incompatible with nucleic acid integrity and can involve specialized chemistry that requires equipment and reagents not normally found in the laboratories of many potential end users of the labelled nucleic acid.

Steric interference by large reporter compounds has been alleviated by the use of linkers, linear chains of, typically four to twelve atoms, usually a saturated or partially unsaturated aliphatic chain, occasionally containing an amide group. The function of linkers has been considered to be to act as a spacer between the nucleotide base and the label. Any linkers capable of providing adequate spacing and flexibility have been considered functionally equivalent.

The use of linkers has also made possible a hybrid labelling technique whereby precursor nucleotides modified to possess a linker moiety are incorporated into the nucleic acid during synthesis, to yield linker-modified nucleic acid. The linker groups suitable for such modification must have a reactive group at the free end of the linker chain. The linker-modified nucleic acid is post-synthetically coupled with a reporter compound at the reactive ends of the incorporated linkers. (See, e.g., Jett et al., U.S. Application Serial Number 07/765,277).

SUMMARY OF THE INVENTION

The present invention is based on the discovery that Jeffamine (TM Texaco, Inc.)-linked nucleoside triphosphates can be substantially superior substrates for incorporating label into nucleic acids during enzyme-catalyzed synthesis. Use of a Jeffamine-linked labelled nucleoside triphosphate derivative as a partial substitute for unlabelled substrate leads to significantly greater incorporation, hence increased label density per unit length of nucleic acid, than the same label joined by a prior art linker. Jeffamine linkers have been found to have the property of permitting higher levels of incorporation of any label.

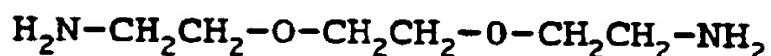
Accordingly, novel Jeffamine-linked deoxy- or ribo-nucleotide derivatives are provided for any sort of nucleic acid label desired: fluorescent, chromatic, bio- or chemiluminescent, ligand or hapten. Similarly, Jeffamine-modified ribo- or deoxyribo-nucleotides are provided for synthesis of Jeffamine-modified nucleic acids. The latter can be post-synthetically modified by attachment of any desired label or combination of labels.

The invention further provides an improved method of synthesizing labelled nucleic acids, whereby greater frequency of label incorporation than heretofore is achieved. Because the Jeffamine-linked deoxy- and ribo-nucleotides of the invention are

incorporated with such high efficiency, the method also provides conventional levels of label using lower amounts of nucleotide derivative in the reaction mixture, thereby providing substantial savings in conventional uses of labelled nucleic acids.

5 DETAILED DESCRIPTION OF THE INVENTION

Jeffamines are polyglycol diamines having a general formula $H_2N-(CH_2)_n-[O-(CH_2)_m]_m-NH_2$, where n is 2 or more, preferably 2 to 4, and m is 1 to 10, preferably 1-5, most preferably 2 or 3. Where m is greater than 1, there can be a different value of n for each $[O-(CH_2)]$ - group. However, typically and most conveniently, the value of n will be the same for each $(CH_2)_n$ group in the compound. Useful Jeffamines of the invention are di-, tri- or tetra- and higher ethylene, n-propylene, or n-butylene glycol diamines. Although Jeffamines having branched $(CH_2)_n$ groups are known, those preferred herein have linear $(CH_2)_n$ groups. The structure of triethylene glycol diamine can be diagrammed as



The choice of any particular Jeffamine is one which can be made readily by those skilled in the art, within limits disclosed herein.

One of the Jeffamine amino groups is used to react with the base moiety of a nucleotide, the other amino group can reacted with a reporter moiety either before or after nucleic acid synthesis. Reaction of a Jeffamine with adenine can be carried out at the 8 position or preferably, at the 6 position of adenine. A Jeffamine derivative of guanine at the 8 position of guanine is the only suitable derivative known. Cytosine can be reacted at the 4 position of cytosine. Jeffamine derivatives of uracil and thymine are only feasible by indirect linkage. Such indirect linkage can be achieved, for example, by first forming allylamine derivatives of the pyrimidine ring at the 5 or 6

position, followed by subsequent reaction with a Jeffamine. Derivatives of cytosine can also be formed in like manner.

The labelled nucleotides of the invention therefore have three parts, the first being a deoxy- or ribo- nucleotide, abbreviated dN or rN (or d or rNTP if in the triphosphate form) which can be either d or r- adenosine (dA or rA), d- or r- guanosine (dG or rG) or d- or r-cytosine (dC or rC). The second part is a Jeffamine-based linker, which will have a chain length of 7 or more atoms, depending on the Jeffamine used, and abbreviated herein as n(J) where n is an integer of 7 or more. The third part is the reporter, designated X. X can be any molecule useful for labeling nucleic acid and having the ability to form stable compounds with a primary amine group. X can be a fluorophore, for example, rhodamine or fluorescein. X can be a chromophore, for example, Nitro blue tetrazolium, or BCIP. X can be a luciferin or other luminescent reporter, such as PPD. X can be a ligand, for example, biotin, having the property of binding another, readily detectable, molecule. Similarly, a ligand such as an enzyme cofactor can be detected by the activity of the enzyme which binds it, the enzyme-catalyzed reaction providing an amplification factor to enhance sensitivity. X can also be a hapten, for example dinitrophenol or digoxigenin, detectable by immunochemical means.

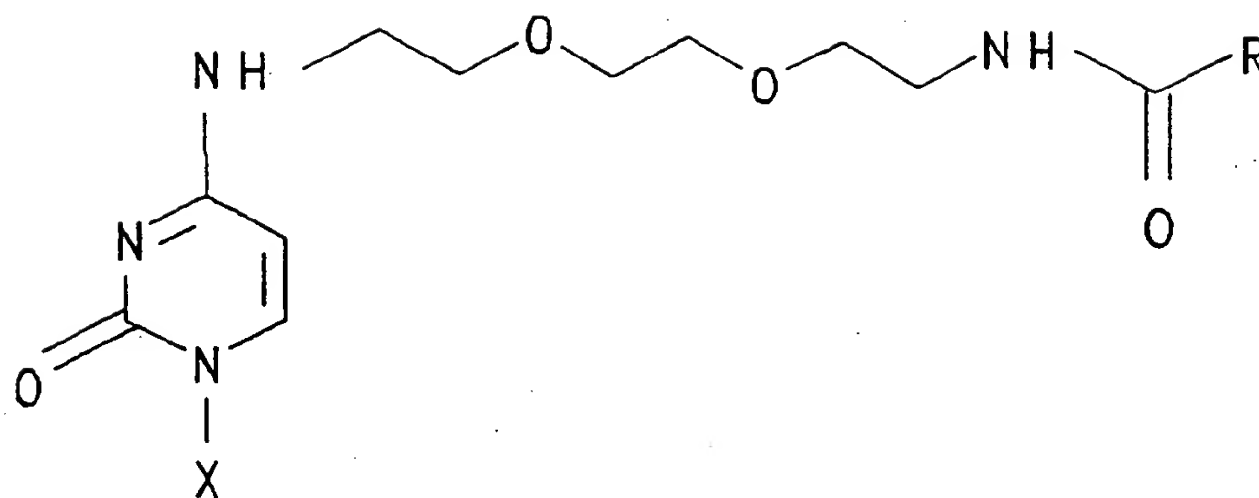
A labelled nucleotide of the invention is therefore abbreviated herein as X-n(J)-(d or r)NTP.

The term "modified nucleotide" is defined herein as a nucleotide having a Jeffamine substituent but no reporter moiety (X is H). A modified nucleotide is useful for post-synthetic labelling of DNA in which the modified nucleotide has been incorporated. Such a modified nucleotide is abbreviated n(J)-(d or r)NTP, where n is 7 or more, and N is adenosine, guanosine or cytidine.

Enzyme-catalyzed addition of labelled nucleotides of the invention to the end of a nucleotide chain can also be accomplished. Using terminal deoxynucleotide transferase, Rho-10(J)-dCTP was successfully added to DNA, using reaction conditions disclosed in U.S. Patent 4,878,979 for end-labelling DNA with Biotin-14-dATP.

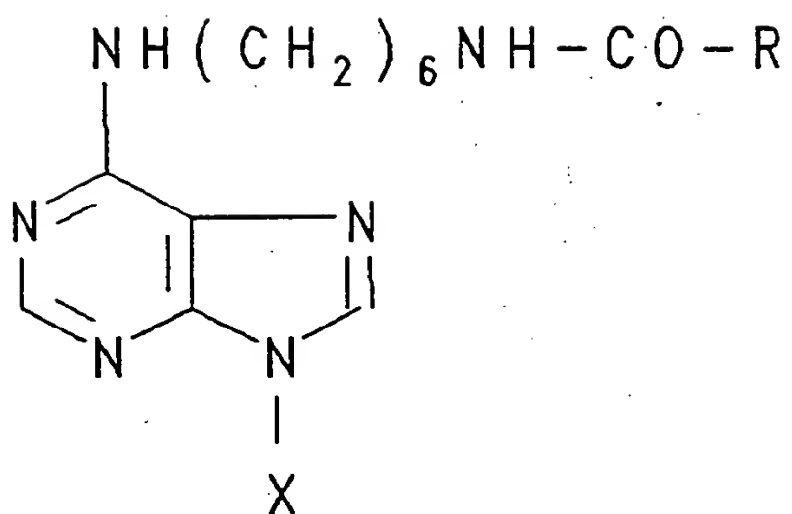
The invention is exemplified by comparing two compounds of the invention, Rhodamine-10(J)-dCTP and Fluorescein-10(J)-dCTP, with prior art compounds, demonstrating surprisingly higher incorporation efficiency than heretofore possible with prior art compounds. The structures of Rhodamine-10(J)-dCTP and the corresponding Fluorescein-10(J)-dCTP are shown in Formula 1. Formulas 2-5 show the structures of Fluorescein- and Rhodamine-8-dATP, Fluorescein- and Rhodamine-4-dUTP, Rhodamine-8-dCTP, Fluorescein-(15)-dCTP, and Rhodamine-(15)-dCTP, respectively.

FORMULA 1



Rho-10(J)-dCTP: R = TETRAMETHYL-RHODAMINE
Fl-10(J)-dCTP: R = FLUORESCEIN
X = DEOXYRIBOSE-5'-TRIPHOSPHATE
OR RIBOSE-5'-TRIPHOSPHATE

FORMULA 2

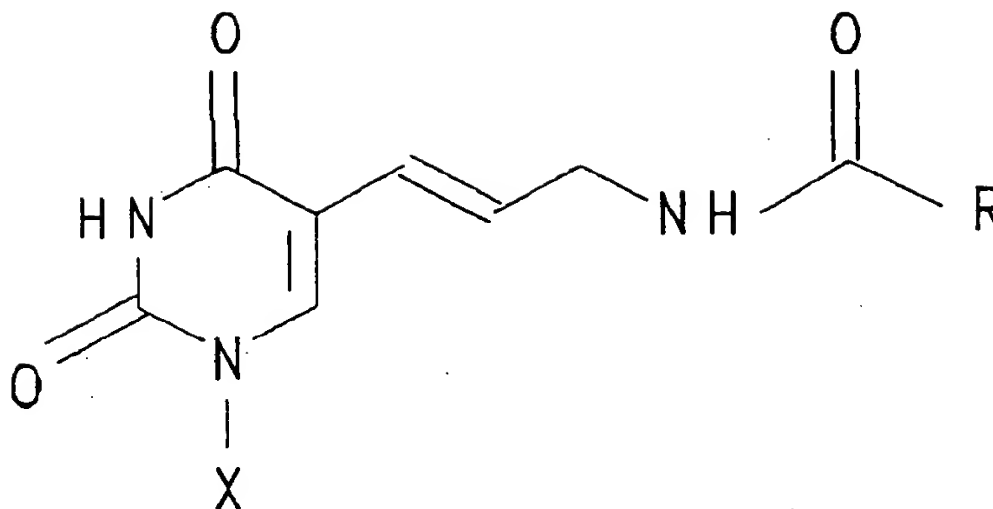


Rho-8-dATP: R = TETRAMETHYL-RHODAMINE

Fl-8-dATP: R = FLUORESCEIN

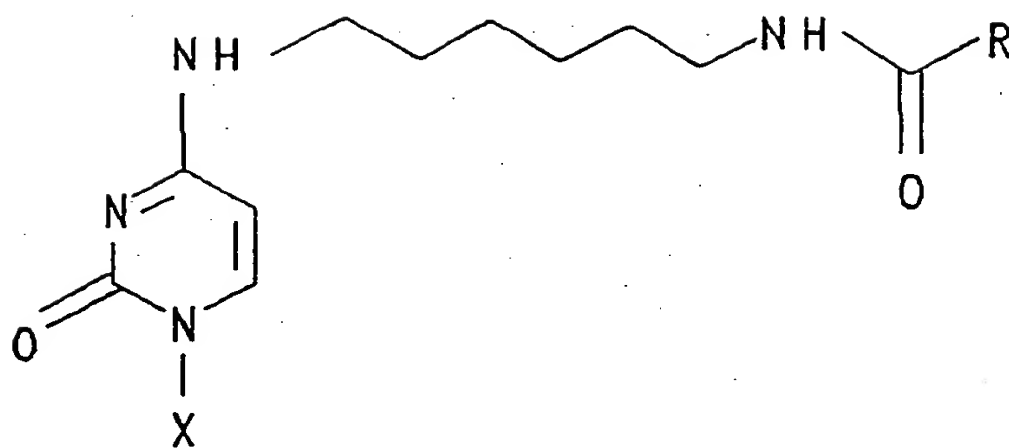
X = DEOXYRIBOSE-5'-TRIPHOSPHATE OR
RIBOSE

FORMULA 3



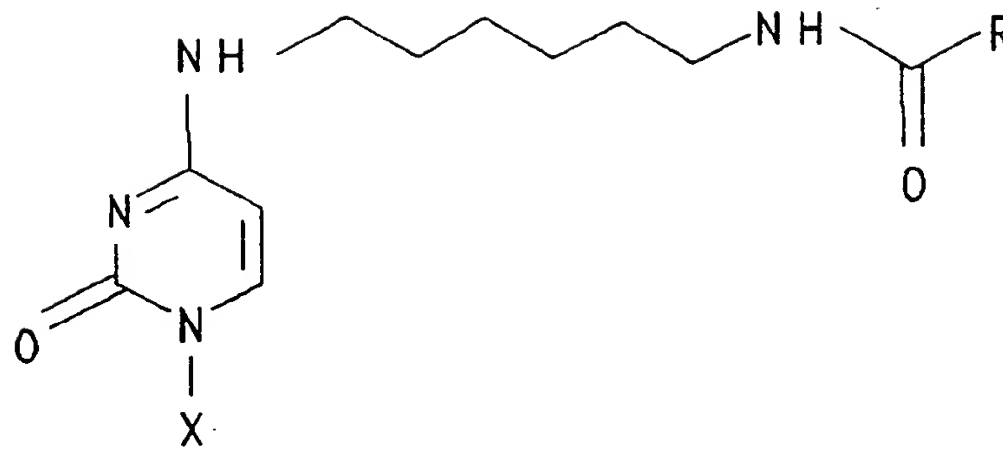
Rho-4-dUTP: R = TETRAMETHYL-RHODAMINE
Fl-4-dUTP: R = FLUORESCEIN
 X = DEOXYRIBOSE-5'-TRIPHOSPHATE

FORMULA 4



Rho-8-dCTP: R = TETRAMETHYL-RHODAMINE

FORMULA 5



Rho-15-dCTP: R = $-(\text{CH}_2)_5\text{NH}-\text{CO}-\text{TETRAMETHYL-RHODAMINE}$
Fl 15-dCTP: R = $-(\text{CH}_2)_5\text{NH}-\text{CO}-\text{FLUORESCEIN}$
X = DEOXYRIBOSE-5'-TRIPHOSPHATE OR
RIBOSE

EXAMPLESExample 1. Preparation of N⁴-triethylene glycol amine-deoxycytidine-5'-triphosphate (10(J)-dCTP)

To triethylene glycol diamine (6.55 mmol, 1 ml) at 0°C was added hydrochloric acid (9.5 mmol, 0.8 ml, 36%) drop-wise. To this solution was added sodium meta-bisulfite (2.2 mol, 430 mg) and water to a final volume of 3 ml. Deoxycytosine-5'-triphosphate (0.1 mmol, 50 mg) was dissolved in the above solution of dimine-bisulfite freshly prepared and filtered. To this solution was added hydroquinone (1 mg in 10 µl of ethanol) and the reaction mixture stirred for 2 days at 45°C under argon. HPLC trace, after adjustment of the aliquot to pH = 8.5, (DuPont Zorbax oligo column, 25% acetonitrile, 0.25 M NH₄H₂PO₄) 2 ml/min. isocratic mode) shows no starting material plus a new major peak (68% of total). The crude mixture was adjusted to pH = 8.5 with sodium hydroxide, diluted to 500 ml with water and loaded on a DEAE anion exchange column equilibrated with triethyl ammonium bicarbonate (TEAB). The column was eluted with a linear gradient of TEAB (0.01 - 1.0 M; 600/600 ml). After desalting of the major peak fractions, 60.6 mg of pure desired product were isolated $\lambda_{\max} = 271-272\text{nm}$.

Example 2. Labelling of amino-nucleoside triphosphates with fluorescent dyes.

The Jeffamine-nucleoside triphosphates (10-20 µmol) e.g., 10(J)-dCTP, were dissolved in sodium bicarbonate (0.4 M, 500 µl) or sodium borate solution (0.1 M) and treated with a 3 to 5-fold molar excess of the N-hydroxysuccinimide ester of the dye (e.g.,

fluorescein or rhodamine) in anhydrous dimethyl formamide (500 μ l). The mixture was reacted for 3 - 18 hr. at room temperature. The reaction was monitored by thin layer chromatography (silica gel; butanol: acetone: acetic acid: 5% ammonium hydroxide: water/70:50:30:30:20) and/or by HPLC. The crude mixtures were diluted in water (200 - 300 ml), loaded on a 10 - 15 cm long, by 1 cm diameter column of mild anion exchange resins and eluted, sequentially, with 0.01, 0.2 and 0.5 M triethylammonium bicarbonate until the fraction containing the fluorescent dNTP was collected. After desalting of the appropriate column fraction, TLC, HPLC and capillary electrophoresis analysis was used to assess the purity and characteristic elution patterns of the desired product. The compounds were characterized by their U.V. spectra as the overlapping spectra of the starting amino modified base and the dyes. Yields of fluorescent nucleotides were 50 - 60%.

Example 3. Use of Rhodamine-10(J)-dCTP in Nucleic Acid Labeling

Five fluorescent nucleotides were initially screened for enzymic incorporation into DNA using random primer extension with Klenow fragment of DNA polymerase I. The five fluorescent nucleotides were fluorescein-8-dCTP, rhodamine-8 dATP, rhodamine-8-dCTP, rhodamine-10(J)-dCTP, and rhodamine-4-dUTP. All but rhodamine-10(J) dCTP (which has two ether linkages) have alkylamine linkers.

Template DNA (100 ng) was denatured in a dilute buffer such as TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) by heating at 100°C for 5 min. Reaction components were added to final concentrations as follows: 50 mM Tris-HCl (pH 6.8), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 400 μ g/ml BSA, 300 μ g/ml random octamers, 200 μ M dCTP, dGTP, and dTTP, 100 μ M dCTP and 100 μ M rhodamine-10(J)-dCTP, 1.0 μ Ci α -[³²P]- dATP (3000Ci/mmol) and 40 units Klenow fragment in a final volume of 50 μ l. After incubation at 37°C for 1-2 hours, 5 μ l 0.2 M EDTA (pH 7.5) was added to terminate the reaction. Incorporation was determined by trichloroacetic acid

(TCA) precipitation. Diluted aliquots from the reaction were spotted on glass fiber filters in duplicate and dried. One of the duplicate filters was washed four times in cold 5% TCA, 20 mM sodium pyrophosphate, then rinsed in 70% ethanol and dried and counted in a liquid scintillation counter (incorporated counts). The second filter was counted directly in the scintillation counter (total counts). Incorporation of radioactive label was 40-50% indicating synthesis of several micrograms of fluorescent DNA probe. When other modified nucleotides were tested a mixture of 100 μ M unmodified dNTP and 100 μ M fluorescent dNTP was used in place of the dCTP/rhodamine-10(J)-dCTP above. When the modified nucleotide was a dATP derivative, α -[32 P]-dCTP was used as a trace label.

TABLE 1

Fluorescent dNTP	% Incorporation	ng DNA Synthesized
Fluorescein-8-dATP	0.6	79
Rhodamine-8-dATP	0.5	66
Rhodamine-8-dCTP	0.1	13
Rhodamine-10(J)-dCTP	42.6	5623
Rhodamine-4-dUTP	0.5	66

The percentage of rhodamine-10(J)-dCTP was varied in subsequent experiments from 75% to 100%. Incorporation decreased with increasing fluorescent nucleotide concentration. Incorporation was increased at higher percentages by increasing the absolute concentration of unmodified dNTP to at least 50 μ M. At a percentage of 95%, incorporation was approximately 15% and at a percentage of 97.5%, incorporation was reduced to about 6%. DNA probes prepared at 50% and 95% both functioned in in situ chromosome hybridizations.

Example 4. Use of Rhodamine-10(J)-dCTP in Nucleic Acid Labeling

Template DNA (500 ng) was denatured in a dilute buffer such as TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) by heating at 100°C for 10 min. Reaction components were added to final concentrations as follows: 50 mM Tris-HCl (pH 6.8), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 400 µg/ml BSA, 300 µm/ml random octamers, 100 µM each of dATP, dGTP, dTTP, and dCTP, 10 µCi α-[³²P]-dATP (3000 Ci/mmol), and 40 units Klenow fragment in a final volume of 50 µl. When testing modified nucleotides the corresponding unmodified dNTP was replaced with 100 µM of the modified nucleoside triphosphates. In some experiments (e.g., Table 2), different mixtures of modified and unmodified nucleotides were used. The percentage of modified nucleotides to the total modified plus corresponding unmodified nucleotide was varied from 25% to 100%. IN all cases, the total concentration of each nucleotide was kept at 100 µM which resulted in a total nucleotide concentration of 400 µM. After incubation at 37°C for 2 hours, 5 µl 0.2 M EDTA (pH 7.5) was added to terminate the reaction. Incorporation was determined by trichloroacetic acid (TCA) precipitation. Diluted aliquots from the reaction were spotted on glass fiber filters in duplicate and dried. One of the duplicate filters was washed four times in cold 5% TCA, 20 mM sodium pyrophosphate, then rinsed in 70% ethanol and dried and counted in a liquid scintillation counter (incorporated counts). The second filter was counted directly in the scintillation counter (total counts). Incorporation of radioactive label was used to determine synthesis of fluorescent DNA probe. When the modified nucleotide was a dATP derivative, α-[³²P]-dCTP was used as a trace label.

TABLE 2

	<u>Fluorescent dNTP</u>	<u>Fluor. dNTP</u>	<u>dCTP</u>	<u>% Incorp</u>	<u>ng DNA synthesized</u>
5	Rhodamine-10(J) - dCTP	100 μ M 75 μ M 50 μ M 25 μ M	--- 25 μ M 50 μ M 75 μ M	5.7 ^a 24.5 33.9 51.8	376 1617 2237 3149
10	Fluorescein-10(J) - dCTP	100 μ M 75 μ M 50 μ M 25 μ M	--- 25 μ M 50 μ M 75 μ M	5.9 ^b 13.8 36.2 54.9	389 911 2389 3623

^a = average of 7 experiments.^b = average of 3 experiments

15 Below, Table 3, is a list of additional modified nucleotides that have been screened for use in enzymatic incorporation into DNA using random primer extension with Klenow fragment of DNA polymerase.

20

TABLE 3

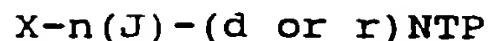
	<u>Fluorescent dNTP</u>	<u>% Incorporation</u>	<u>ng DNA Synthesized</u>
	Fluorescein-8-dATP	1.2	79
	Rhodamine-8-dATP	1.8	119
25	Rhodamine-8-dCTP	0.49	32
	Rhodamine-10(J) -dCTP	5.7 (avg of 7)	376
	Fluorescein-10(J) -dCTP	5.9 (avg of 3)	389
	Fluorescein-(15) -dCTP	1.5	148
	Fluorescein-4-dUTP	1.7	112
30	Rhodamine-(12) -dUTP	12	825

Similar experiments were also performed using T5 DNA polymerase. The reaction conditions were identical to those of Example 4 (Table 2) except that T5 DNA polymerase was used and

the buffer composition for the reaction was 50 mM HEPES (pH 7.3), 10 mM $MgCl_2$, 50 mM ammonium sulfate, 5 mM DTT. It was found that using Rhodamine-10(J)-dCTP and Fluorescein-10(J)-dCTP as the modified nucleotides, 7.8% and 7.4% of nucleotides were incorporated into TCA precipitable material, respectively. This corresponds to 515 and 488 ng of total DNA synthesized, for Rhodamine-10(J)-dCTP and Fluorescein-10(J)-dCTP respectively.

CLAIMS

1. A modified nucleotide compound having the formula



where N is adenosine, guanosine or cytidine; X is H, a fluorophore, a chromophore, a luminescent compound, a ligand or a hapten; and n is an integer of 7 or more.

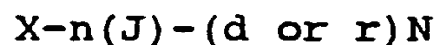
2. A compound according to claim 1 wherein X is H, fluorescein, rhodamine, nitroblue tetrazolium, BCIP, firefly luciferin, PPD, biotin, dinitrophenol or digoxigenin.

3. A compound according to claim 1 wherein X is rhodamine, N is cytidine and n is 10.

4. A compound according to claim 1 wherein X is Fluorescein, N is cytidine and n is 10.

5. A compound according to claim 1 wherein X is biotin, and n is 10.

6. Nucleic acid comprising a modified nucleotide of the formula



where N is adenosine, guanosine or cytidine; X is H, a fluorophore, a chromophore, a luminescent compound, a ligand or a hapten; and N is an integer of 7 or more.

7. The nucleic acid of claim 6 wherein the nucleic acid is DNA and N is dA, dG or dC.

8. The nucleic acid of claim 6 wherein the nucleic acid is DNA, N is dA, dG or dC and X is H, Fluorescein, rhodamine, nitroblue tetrazolium, BCIP, firefly luciferin, PPD, biotin, dinitrophenol or digoxigenin.

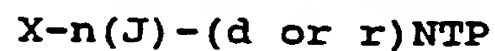
9. The nucleic acid of claim 6 wherein the nucleic acid is DNA, N is dC, X is fluorescein or rhodamine and n is 10.
10. In a method of nucleic acid synthesis by a reaction catalyzed by a nucleic acid polymerase enzyme wherein the polymerase is present in a reaction mixture comprising a template nucleic acid and one or more deoxy- or ribo-nucleotide triphosphates, the improvement comprising substituting for a portion of one of said nucleotide triphosphates X-n(J)-(d or r)NTP where N is adenosine, guanosine or cytidine; X is H, a fluorophore, a chromophore, a luminescent compound, a ligand or a hapten; and n is an integer of 7 or more.
11. The method of claim 10 wherein the enzyme is a DNA polymerase, the reaction mixture comprises deoxynucleotide triphosphates and X is H, fluorescein, rhodamine, nitroblue tetrazolium, BC1P, firefly luciferin, PPD, biotin, dinitrophenol or digoxigenin.
12. The method of claim 10 wherein the enzyme is a DNA polymerase, the reaction mixture comprises deoxynucleotide triphosphates, X is fluorescein or rhodamine, N is C and n is 10.
13. The method of claim 10 wherein the enzyme is the Klenow fragment of E. coli DNA polymerase, the reaction mixture comprises deoxynucleotide triphosphates, X is fluorescein or rhodamine, N is C and n is 10.
14. The method of claim 10 wherein the enzyme is T5 polymerase, the reaction mixture comprises deoxynucleotide triphosphates, X is fluorescein or rhodamine, N is C and n is 10.
15. The method of claim 10 wherein the enzyme is terminal deoxynucleotide transferase, the reaction mixture comprises

deoxynucleotide triphosphates, X is fluorescein or rhodamine, N is C and n is 10.

- 5 16. A kit for making a fluorescent nucleic acid having four ribo- or deoxynucleotides in its composition and having at least one of the ribo or deoxynucleotides partially substituted by a labelled ribo- or deoxynucleotide, comprising:

a) a nucleic acid synthesizing enzyme;

10 b) a modified nucleotide compound having the formula



where N is adenosine, guanosine or cytidine; X is H, a fluorophore, a chromophore, a luminescent compound, a ligand or a hapten; and n is an integer of 7 or more.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/02390

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 15/12

US CL :536/23.1, 25.3, 25.32, 25.6; 435/91

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 25.3, 25.32, 25.6; 435/91

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, WORLD PATENTS, BIOSIS, APS

Key words: Jeffamine, linker, spacer, fluorescein, rhodamine, non-radioactive labeling of DNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,914,210 (Levenson et al) 03 April 1990, see column 5.	1-16
Y	US, A, 4,962,029 (Levenson et al) 09 October 1990, see columns 4 and 5.	1-16
Y	US, A, 4,828,979 (Klevan et al) 09 May 1989, see column 6.	1-16
Y	Proceedings National Academy of Sciences, USA, Volume 78, No. 11, issued November 1981, P. R. Langer et al., "Enzymatic Synthesis of Biotin-Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes", pages 6633-6637, see entire document.	1-16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Methods in Molecular and Cellular Biology, Volume 3, issued February 1992, H. Voss et al., "New Procedure for Automated DNA Sequencing with Multiple Internal Labeling by Fluorescent dUTP", pages 30-34, see especially page 33.	1-16
Y	Journal of Cellular Biochemistry, Supplement 16B, issued February 1992, M. L. Hammond et al., "Enzymatic Synthesis and Exonucleolytic Degradation of Fluorescent DNA Containing Rhodamine and Fluorescein Nucleotides", page 46, abstract F 325, see entire abstract.	1-16
Y,P	Electrophoresis, Volume 13, issued September 1992, W. Ansorge et al., "High Throughput Automated DNA Sequencing Facility with Fluorescent Labels at the European Molecular Biology Laboratory", pages 616-619, see especially page 617.	1-16

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